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COTTON OVULE CULTURE: A TOOL FOR BASIC BIOLOGY, BIOTECHNOLOGY AND COTTON IMPROVEMENT

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Summary

Nearly 30 years ago the conditions for culturing immature cotton ovules were established to serve as a working research tool for investigating the physiology and biochemistry of fiber development. Not only has this tissue culture method been employed to characterize the biochemistry of plant cell expansion and secondary cell wall synthesis, but ovule cultures have contributed to numerous other aspects of plant cell physiology and development as well. In addition to basic studies on fiber development, cotton ovule cultures have been used to examine plant–fungal interactions, to model low temperature stress responses, to elucidate the pathways responsible for pigment formation in naturally pigmented fiber and to probe how cytoskeletal elements regulate cell wall organization. Success in rescuing *Gossypium* interspecific hybrids was dependent on ovule culture media formulations that could support early embryo development *in ovulo*. As tissues produced in culture are analyzed by increasingly more sophisticated techniques, there appear to be some differences between ovule growth *in planta* and ovule growth *in vitro*. Discerning how ovule culture fiber development is different from fiber development in field-grown plants can contribute valuable information for crop improvement. Cotton ovule cultures are an especially attractive model system for studying the effects of gravity on cell elongation, cellulose biosynthesis and embryo development and are excellent targets for examining transient expression of introduced gene constructs. With only minor modification, the procedure originally described by C. A. Beasley and I. P. Ting for growing cotton ovules *in vitro* will continue to be a useful research tool for the foreseeable future.

Key words: cellulose; cell wall; cotton fiber; cottonseed; embryo; *Gossypium*.

Cotton Ovule Culture: Early History

Since the earliest beginnings of plant physiology as a discipline, plant scientists have been seeking to identify the essential nutritional and hormonal components required for plant growth and development. Interest in establishing culture conditions to support the growth of cotton ovules arose for two reasons. First, if the phytohormone requirements for fiber production *in vitro* could be identified, then it might be possible to improve cotton fiber yield, quality and uniformity by treating cotton fields with exogenous plant growth regulators. Secondly, success with *in vitro* embryo development with other plants suggested that it might be possible to rescue cotton interspecific hybrids that had proven to be unattainable by any other means.

In the 1950s and early 1960s there were a number of attempts to grow cotton embryos in culture (Dure et al., 1957; Lofland, 1950; Mauney, 1961; Mauney et al., 1967; Weaver, 1957). All of the investigators were successful in obtaining seedlings from immature embryos at a low frequency when the embryos were excised 20–25 d post-anthesis (DPA); however, younger embryos failed to mature properly *in vitro.* As embryo abortion in interspecific crosses nearly always results by 7–14 DPA (Mauney, 1961), clearly new approaches were needed for embryo rescue.

C. A. 'Bud' Beasley, a young researcher at the Southern California Laboratories of the Stanford Research Institute, was working on two projects in the late 1960s. The first was to clonally propagate plantlets from a woody species that was extremely difficult to grow vegetatively. The second project, sponsored by the progenitor of Cotton Incorporated, required growing cotton plants under greenhouse and growth chamber environments. With the ready availability of flowering cotton plants and facilities for tissue culture, Beasley tried a few preliminary experiments to culture isolated cotton ovules. He found that, when a liquid medium was supplemented with coconut milk (water), the young ovules survived for a longer period of time than when coconut milk was absent from the medium. This promising result was the foundation for a grant proposal to Cotton Incorporated that was submitted through the University of California, Riverside, in association with Dr. I. P. Ting. In March 1970 work began in earnest to find the optimal conditions for cotton ovule culture that would support both embryo growth and fiber development.

Many of the published recipes for tissue culture media at the time were low salt formulations such as White's (White, 1957) and Nitsch's (Nitsch, 1951) media. Initially, cotton ovules were intentionally submerged in low salt, liquid media, but these conditions failed to support ovule development. Experiments with

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Fig. 1. C. A. 'Bud' Beasley on the cover page of the October 1971 issue of *California Agriculture* announcing success in growing cotton *in vitro*.

solidifying media with agar were equally unsuccessful, resulting in callus formation and excessive phenolic browning of the ovules. Finally, by October 1971, after numerous combinations of inorganic nutrients, carbohydrate sources and plant growth regulators, a short article in *California Agriculture* was published announcing success in growing 'test tube cotton' (Beasley et al., 1971) (Fig. 1). The best combination of conditions used fertilized ovules floating on the surface of a liquid high salt medium. The basal medium was modified from the Murashige and Skoog formulation (Murashige and Skoog, 1962) in two important ways: nitrogen was in the form of KNO_3 instead of NH_4NO_3, and a mixture of glucose and fructose was substituted for sucrose. At approximately the same time, a Ph.D. student at the University of Ghent, Belgium, reported that fertilized cotton ovules from 10 DPA would develop normal embryos and produce some fiber when grown in liquid Murashige–Skoog medium (Delange and Eid, 1971; Eid, 1972; Eid et al., 1973).

Further refinements in the phytohormone regimes resulted in the publication of optimized procedures for culturing fertilized (Beasley and Ting, 1973) and, subsequently, unfertilized (Beasley and Ting, 1974) cotton ovules. Fertilized ovules (2 DPA) do not require hormones and exogenous auxin [indole-3-acetic acid (IAA)] does not promote additional fiber growth. Addition of exogenous gibberellic acid (GA_3), however, will promote supplementary fiber production in fertilized ovules and kinetin and abscisic acid (ABA) are inhibitory (Beasley and Ting, 1973). In the case of unfertilized ovules, IAA promotes more fiber production than GA alone, and together their effect on fiber development is additive (Fig. 2). Exogenous kinetin promotes an increase in the size of unfertilized ovules but does not alone support fiber growth. Abscisic acid is inhibitory to ovule and fiber development in unfertilized ovules (Beasley and Ting, 1974).

To compare the effect of different culture conditions on the production of fiber *in vitro*, a rapid screening method was developed using the stain Toluidine Blue O (TBO) (Beasley et al., 1974). In this semi-quantitative procedure for measuring fiber production, ovules were stained with TBO for a short period of time, washed to remove excess stain, and then destained with an ethanol–acetic acid solution. The absorbance of the destaining solution was measured and compared with an arbitrarily defined absorbance value that is defined as one total fiber unit (TFU). Groups of ovules may be stained and destained simultaneously as described by the original work (Beasley et al., 1974) or individual ovules may be destained to assess the variation in TFU values for a particular culture treatment (Triplett, 1998).

The initial objective to find 'a working research tool to investigate the physiology and biochemistry of fiber development' (Beasley et al., 1971) was certainly met within the first 3 yr of this project. Until 1977 Beasley continued to collaborate with many investigators eager to use cultured cotton ovules to investigate various aspects of plant biochemistry. Ovule cultures were used to compare UDP-glucose with GDP-glucose as substrates for cellulose biosynthesis (Delmer et al., 1974), to examine the influence of boron on cell wall metabolism (Birnbaum et al., 1974, 1977), to study osmoregulation of fiber elongation by potassium and malate (Dhindsa et al., 1975), to analyze a potential interaction between phytohormones and nitrogen metabolism (Beasley, 1977; Beasley et al., 1979) and to investigate the morphology of embryo sac development immediately prior to fertilization (Jensen et al., 1983).

Variations in ovule culture performance between different bolls and between experiments was a constant problem (Beasley, 1992). Beasley attributed these problems to subtle differences in environmental conditions that could not be controlled. To a large extent the variation in ovule development between bolls can be overcome by randomly selecting ovules from several bolls for each culture treatment and by growing ovule cultures in an enriched CO_2 environment (Xie and Stewart, 1989).

Hybrid Embryo Rescue

Worldwide there are four species of cultivated cotton: these include diploids from the Old World, *Gossypium herbaceum* and *Gossypium arboreum*, and the New World tetraploid species *Gossypium hirsutum* and *Gossypium barbadense*. In addition, there are over 30 wild species of cotton that have been collected to preserve disease/insect resistance genes and other useful traits for introduction into the commercially important species. Interspecific crosses in the genus *Gossypium* often lead to a variety of developmental failures, all resulting in early embryo abortion.

In spite of numerous attempts to produce viable seedlings from interspecific crosses by culturing embryos or immature ovules, the frequency of success was always limited (Dure et al., 1957; Lofland, 1950; Mauney, 1961; Mauney et al., 1967; Weaver, 1957). In the first informal report on the cotton ovule culture system, Beasley and co-authors included a photograph of a well-developed seedling produced from a 2 DPA ovule after 2.5 mo in culture (Beasley et al., 1971). Expanding on this approach, Stewart and Hsu (1977, 1978) tried the Beasley–Ting (BT) basal medium supplemented with phytohormones and found that embryo development in self-fertile

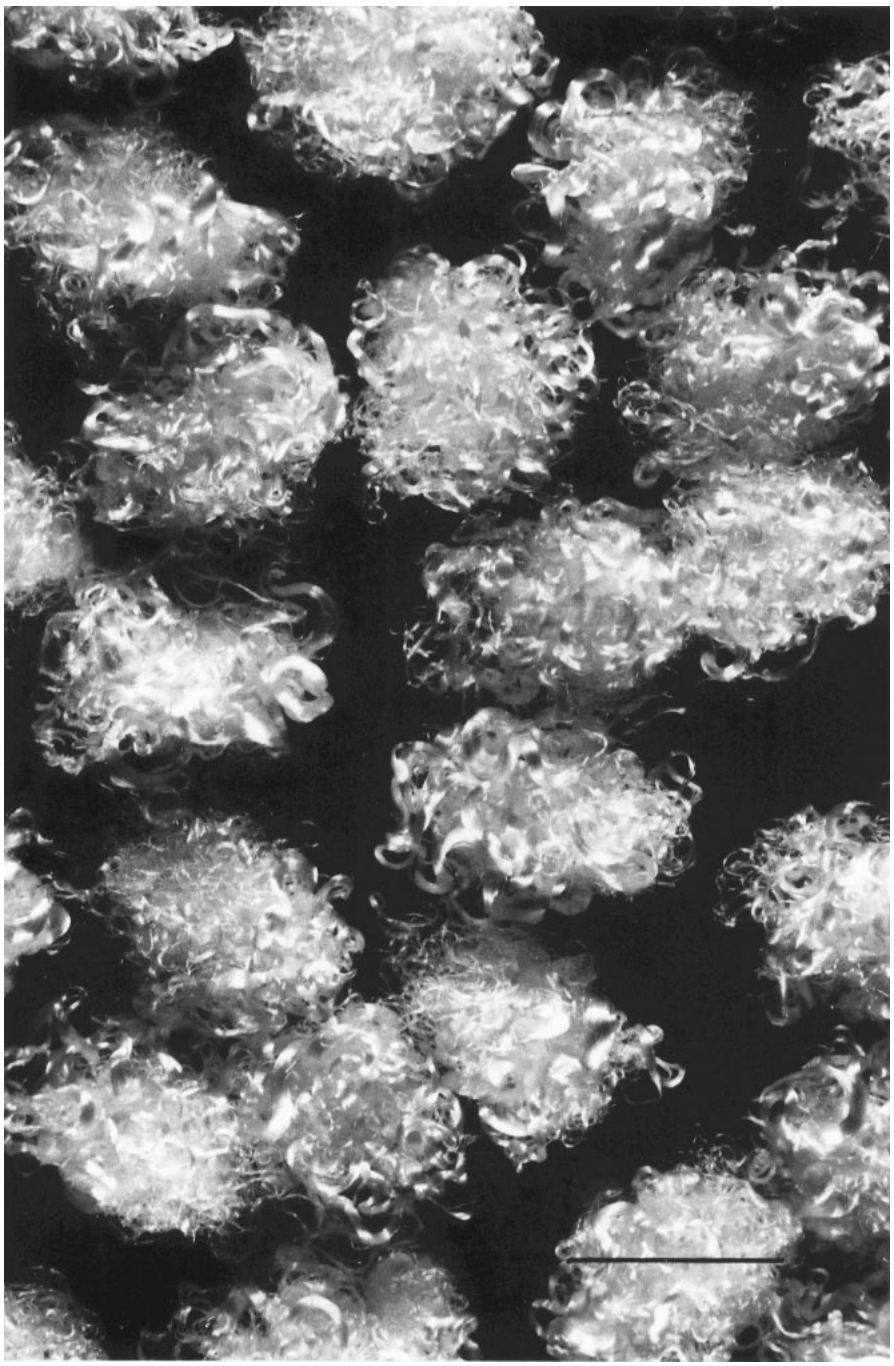

Fig. 2. Cotton ovule cultures at 16 d post-anthesis. Scale bar = 10 mm.

embryos was normal up to the early cotyledon stage but that subsequent development was impeded. Noting that the capacity of young cotton ovules to reduce nitrogen was insufficient to support cotton ovule development (Radin and Sell, 1975), and that the ammonium form of nitrogen was necessary for somatic embryogenesis in wild carrot (Ammirato and Steward, 1971), Stewart and Hsu tested varying amounts of ammonium salts in the basal BT medium. A supplement of 10–15 mM NH_4Cl or NH_4NO_3 supported the development of the greatest number of ovules to maturity. Typically, Beasley cultured ovules for 2–3 wk before evaluating the extent of fiber growth. Stewart and Hsu planned to culture ovules for much longer culture periods and therefore the carbohydrate source was changed from ~2% (w/v) glucose in the BT medium to 4% (w/v) sucrose in the modified Stewart–Hsu medium. These changes to BT medium supported the growth of hybrid embryos derived from a cross between *G. hirsutum* (♀) with *G. arboreum* (♂) (Stewart and Hsu, 1978). The yield of embryos that fully developed under these conditions was highest, slightly over 50%, when phytohormones were omitted. For other interspecific crosses, the yield of embryos was lower and was dependent on the presence of phytohormones

(Stewart and Hsu, 1978). Embryo germination on White's medium adjusted to pH 7.0 resulted in the highest number of viable seedlings (Stewart, 1979). Embryo development in ovules from the diploid species was more difficult to produce *in vitro* than in tetraploid ovules. Nevertheless, Stewart and Hsu were able to rescue several interspecific hybrids between the four cultivated species and wild species of *Gossypium* using ovule culture (Stewart, 1981). Using three wild species as the female parent, Umbeck and Stewart (1985) were able to recover interspecific hybrids from eight of the 12 crosses that they made using the four cultivated species as male parents. Recovering these interspecific hybrids between wild and cultivated species, especially when the maternal parent is a wild species, permits introgression of mitochondrial and chloroplast genes from wild species into breeding lines and commercial cultivars (Stewart, 1991, 1992).

Fiber Development

Many of the known details about cotton fiber development have arisen from studies of fibers growing on immature cotton ovules in culture. Fibers originate from the epidermal layer of cells on the ovule and elongate until each fiber cell is over 1 cm long when grown *in vitro*. Secondary wall synthesis commences before cell elongation has ceased. Unlike secondary walls in most higher plants, cotton fiber secondary walls are nearly pure, highly crystalline, cellulose and contain no lignin.

Before the procedure for culturing ovules could enjoy widespread use as a tool for studying fiber development, the fiber produced by ovule cultures needed to be compared, in as many ways as possible, with fiber developed *in planta*. When such a study was undertaken, the fibers produced *in vitro* were found to be quite similar to fibers produced on the plant (Meinert and Delmer, 1977). Although the fiber cells in culture do not grow to the same final length as fibers developed on plants, the cellulose content of the cell wall and the neutral sugar and uronic acid compositions of plant-grown and culture-grown fibers were found to be remarkably similar throughout fiber development. The onset of secondary wall synthesis was earlier for *in vitro*-grown fibers than for plant-grown fibers for what are still unknown reasons. It is possible that there is a relationship between the early initiation of secondary wall synthesis in cultured fibers and the early cessation of fiber elongation leading to shorter fibers. Little is known about the developmental cues underlying the transition period in fiber development when cell elongation and secondary wall synthesis are both occurring. Understanding the molecular basis for why *in vitro*-produced fibers are shorter than plant-derived fibers and why cellulose biosynthesis in the secondary wall begins earlier *in vitro* can lead to improvements in cotton fiber quality, as the timing of the transition period dictates fiber length and cell wall thickness.

For the first 5 d after anthesis, nuclear and nucleolar volumes increase in fiber cells grown *in planta*; a similar increase in size is observed *in vitro* only if auxin and GA_3 are present (Kosmidou-Dimitropoulou, 1980, 1986). By 5 DPA the amount of DNA in each fiber nucleus increases by 24% (6.8 pg/nucleus) from the amount of DNA present at 2 DPA (van't Hof, 1999). Currently, the identity of DNA sequences that are amplified are unknown; however, due to the concomitant increase in nucleolar size it is likely that amplification involves the ribosomal RNA genes.

One of the major advantages of using ovule cultures to study fiber development is that the effects of inhibitors on fiber initiation and growth or incorporation of radiolabeled compounds can be monitored. As a result, small amounts of potentially toxic, expensive or rare compounds can be evaluated at reduced cost and without risking workers or the environment to exposure. Ovule cultures have been treated with a DNA replication inhibitor, auxin transport inhibitor and GA_3 biosynthesis inhibitor (Dhindsa, 1978), cellulose biosynthesis inhibitors (Montezinos and Delmer, 1980; Francey et al., 1989), lipid-linked glycosyl transferase inhibitor and Na^+ ionophore (Davidonis, 1993), cytoskeleton disrupting agents (Seagull, 1990) and a transcription inhibitor (Triplett, 1998). Adding alpha–amanitin at concentrations to inhibit RNA polymerase II prevented the normal progression of fiber development depending on when the inhibitor was added. The inhibition was reversible during the early stages of fiber initiation and cell elongation, but irreversible if ovules were left in contact with alpha–amanitin for greater than 5 d. This observation suggests that there is a window of opportunity for fiber cell initials to develop into elongated fiber cells. If fiber cells do not differentiate within 4–6 d post-anthesis, fibers lose their capacity to differentiate.

At least three differences between fiber development *in vitro* and *in planta* have been revealed in recent years. In one study the cell wall polymer molecular weight distributions from fibers grown *in vitro* were compared with the profiles from mature and immature plant-grown fibers (Triplett and Timpa, 1995). The method involved dissolving fibers in a solution of dimethylacetamide–lithium chloride and separating the cell wall polymers by size exclusion (gel permeation) chromatography (Timpa, 1991). The cumulative molecular weight distributions of ovule culture fibers after 21 d in culture were similar to measurements for 30 DPA plant-grown fibers, but ovule culture fibers lacked a low molecular weight polymer fraction. In another study comparing protein profiles between culture-grown ovules and plant-grown ovules, four small molecular weight acidic proteins between 14 and 19 kDa were tenfold more abundant in cultured ovules than field-grown ovules (Turley, 1998). The identity of these proteins is unknown at present. In the third instance for which cultured fibers were shown to exhibit anomalous properties, up to 25% of fiber cells were shown to be multicellular when fertilized ovules (2 DPA or older) were cultured in the absence of auxin (van't Hof and Saha, 1997, 1998). In the same study multicellular fibers were never seen growing on ovules sampled from plants. Taken together these three instances show that there are subtle differences in ovule and fiber development *in vitro* compared with *in planta*, and care must be exercised when extrapolating results from the tissue culture incubator to the field.

Fiber Cytoplasts

In spite of the abundance of cellulose in the biosphere, isolation of enzyme activities responsible for cellulose biosynthesis has proven quite challenging over the years (Haigler and Blanton, 1996). In looking for model systems to monitor the use of sugar nucleotides as substrates for cell wall polymer synthesis, protocols were developed to isolate protoplasts from cotton ovule epidermal cells (Gould et al., 1986a). As cellulose synthesis is most active 14–16 d after anthesis when cotton fiber cells are nearly 2.5 cm long, Gould and co-workers reasoned that protoplasts from developing fibers of this stage might retain more cellulose biosynthetic activity than protoplasts from day-of-anthesis ovules.

In fact, fiber cells from cotton ovule cultures were a better starting tissue for producing anucleate protoplasts (cytoplasts) than fibers collected from greenhouse-grown plants (Gould et al., 1986b). Ovules with attached fibers were harvested from cultures and treated with a mixture of cell wall degrading enzymes. Cytoplasts were separated from subcellular debris and other contaminants by centrifugation on Ficoll density gradients. Cytoplasts cultured in BT medium supplemented with a revised complement of phytohormones remained viable for at least a week as judged by fluorescein diacetate staining and $^{14}CO_2$ evolution. Cell division did not occur during this time period but, within 2 h after isolation, cytoplasts produced material on the cell surface that stained with Tinopal LPW (Calcofluor White), a stain for β-glucans. Cell wall polymer deposition continued for at least a week with the majority of [^{14}C]-glucose being incorporated into glucans and lipids. Analysis of the glucan fraction by gas chromatography-mass spectrometry revealed that, when UDP [^{14}C]-glucose was the substrate, the predominant glucans were β-1,3-glucans (callose) and only a small amount of cellulose, a β-1,4-glucan. Interestingly, when [^{14}C]-glucose was provided as a substrate, the majority of the labeled glucan polymers were β-1,4-glucan. The amount of cellulose produced during wall regeneration by fiber cytoplasts was comparable to the levels found in fiber primary cell walls. Therefore, even though cytoplasts were prepared from ovule-culture fiber cells when cellulose synthesis was very active, continuation of this enzyme activity did not persist in isolated cytoplasts.

Fiber Cytoskeleton

The orientation of cellulose microfibrils in the cell wall is an important determinant of fiber tenacity. The arrangement of cellulose polymers in the cotton fiber wall parallels the organization of the cortical cytoskeleton (Seagull, 1993). When cotton ovule cultures were treated with colchicine, trifluralin or oryzalin, to disrupt microtubules, or cytochalasins B and D, to depolymerize microfilaments, cellulose microfibril organization in fiber cells was altered (Seagull, 1990). Ovule culture-grown fibers were also used in a study to quantify the changes in orientation, length, number and subcellular location of microtubules during fiber development. The main reason why fibers produced *in vitro* are preferable to plant-grown fibers for immunofluorescence studies is that fibers from adjacent ovules in the boll (carpel) become entangled only a few days after anthesis. With older samples where the fibers are quite long the problem with entanglement is significant. The plant-grown ovules must be teased apart for analysis, risking the possibility of fiber damage. As cultured ovules are floating on the surface of the medium there is little opportunity for fibers to intertwine *in vitro*.

In higher plants, little is known about the mechanisms through which the cytoskeleton influences cellulose microfibril organization. Based on studies in animal cells, it is believed that proteins that associate specifically with the cytoskeleton exert an influence on its organization. In order to characterize cotton fiber cytoskeleton-associated proteins and study their influence on cellulose deposition, a procedure for isolating these proteins had to be devised. Once again, ovule cultures proved to be an excellent starting material for making anucleate protoplasts. Cytoplasts were extracted with the detergent Triton X-100 to remove the plasmalemma and other noncytoskeleton proteins and purified on a sucrose step gradient (Andersland et al., 1998). The extracted cytoplast preparation was enriched in alpha- and beta-tubulin and actin, but contained no other major protein component.

Immunological techniques were used to determine if two proteins, previously shown to be associated with plant cytoskeletons, were present in cotton fiber cytoskeleton preparations. Specifically, neither elongation factor 1-alpha (Durso and Cyr, 1994) nor spectrin (Yang et al., 1992) was present in isolated cytoskeletons. These results suggest that the major components of cotton fiber cytoskeleton are the two tubulins and actin, and that associated proteins are present in much smaller proportions than in other plant cells for which this type of analysis has been performed. Recently, a 115 kDa protein has been identified from isolated cytoskeleton preparations that appears to associate specifically with microfilaments and may represent a true actin-associated protein in cotton fiber (Andersland and Triplett, 2000).

Cotton ovule cultures have also been used to show that the stability of the cytoskeleton in ovule epidermal cells at anthesis may influence the number of cotton fiber cells that are initiated (Seagull, 1998). The number of newly initiated fiber cells was determined by macerating the ovule tissue in a fixative and counting the fibers under low magnification (van't Hof, 1998; van't Hof and Saha, 1998). Agents that disrupted microtubules or microfilaments resulted in fewer fiber cells being initiated, while taxol, a microtubule stabilizing agent, resulted in 20% more fiber initials.

Low Temperature Stress

Suboptimal night-time temperatures have a negative influence on cotton fiber quality, yield and, hence, crop value. In an effort to understand the physiological basis for alterations in fiber growth, cotton ovule cultures were used to examine the effects of cool temperature on fiber development (Haigler et al., 1991; Seagull, 1998; Xie et al., 1993). For studies such as these, the ability to produce fibers *in vitro* under defined environmental conditions permits many different cultivars to be tested under multiple temperature regimes and in a relatively small area. Maintaining ovule cultures at a constant temperature (34°C) resulted in fiber secondary walls with uniformly smooth morphology. Diurnal cycling of the temperature between 34°C and 22°C or 15°C resulted in 'growth rings' appearing in cross-sections of fiber cell walls (Haigler et al., 1991). The appearance of these rings was similar to the morphology of fiber cross-sections from field-grown plants exposed to low night temperatures. Respiration rates in cotton ovule cultures were also sensitive to lower temperatures; however, recovery of cellulose biosynthesis was on a different timescale than recovery of respiration when cultures were returned to warmer temperatures (Roberts et al., 1992). When ovule cultures were initiated at low temperatures, both fiber initiation and early phases of cell elongation were independently delayed compared with ovule cultures grown at a constant 34°C (Xie et al., 1993). Later stages of fiber cell elongation were temperature-independent. When the early phases of fiber cell elongation were slowed down by low temperature exposure, the overall period for the later stages of cell expansion increased. Collectively, these experiments show that the physiological response to low temperature by growing fibers is independent of the whole plant, and that the cotton ovule culture system is an appropriate model for research on the physiology/biochemistry of cotton fiber response to low temperature.

TABLE 1

COTTON OVULE GROWTH AND MEDIA FINAL PH WHEN OVULES WERE GROWN ON BT MEDIA WITH SORBITOL AS AN OSMOTIC AGENT

Treatment	Media ψ_π [a] (bars)	Dry weight[b] (mg ovule^{-1})	Media pH [c]
100 m*M* glucose 0 m*M* sorbitol	−2.54	12.80 ± 2.2	5.42 ± 0.03
100 m*M* glucose 100 m*M* sorbitol	−5.08	10.13 ± 1.8	5.81 ± 0.28
100 m*M* glucose 200 m*M* sorbitol	−7.62	8.45 ± 2.1	6.24 ± 0.35
100 m*M* glucose 300 m*M* sorbitol	−10.16	5.99 ± 0.7	6.38 ± 0.37
200 m*M* glucose 0 m*M* sorbitol	−5.08	14.7 ± 2.6	5.53 ± 0.07
200 m*M* glucose 100 m*M* sorbitol	−7.62	13.12 ± 1.7	5.51 ± 0.0
200 m*M* glucose 200 m*M* sorbitol	−10.16	11.29 ± 0.8	6.31 ± 0.52
200 m*M* glucose 300 m*M* sorbitol	−12.70	9.33 ± 1.1	6.58 ± 0.31

[a] $\psi_\pi = -miRT$, where m = molality of solute/1000 ml H_2O, i = activity constant, R = gas constant and T = absolute temperature (K).

[b] *Gossypium hirsutum* (Texas Marker-1) unfertilized ovules after 21 d in culture.

[c] Culture medium was adjusted to pH 6.0 at the start of the culture period and measured after the ovules were harvested at 21 d in culture.

Osmotic Stress

In addition to temperature stress, other abiotic stress responses lead to severe reductions in cotton fiber yield. Cotton ovule cultures appear to be a suitable model system for examining the physiological and molecular bases of osmotic stress responses. Addition of osmotic agents such as sorbitol to BT media resulted in inhibition of ovule development and differences in the media pH at the end of the culture period (Table 1). Additional analyses of these cultures are pending (Triplett, unpublished work).

Plant–fungal Interactions

Aflatoxin, a secondary metabolite produced by the fungus *Aspergillus flavus,* is a potent hepatocarcinogen (Payne, 1992). Designing ways to prevent *A. flavus* infection of cotton and other crops, thereby reducing aflatoxin contamination, is an important post-harvest research activity. Concomitant with *Aspergillus* infection, a yellow–green fluorescent compound derived from the peroxidase-catalyzed oxidation of kojic acid is produced in cotton bolls (carpels). Kojic acid is produced by the fungus and the peroxidase activity is produced by the plant host. Fungal infection of developing cotton bolls results in higher levels of peroxidase activity in developing boll tissues. When cotton ovule cultures are inoculated with *A. flavus*, developing ovules respond in a similar manner, producing large amounts of peroxidase in the culture medium (Mellon, 1986). In fact, a group of anionic peroxidases form the most abundant proteins produced in filtrates of culture medium (Mellon, 1991; Mellon and Triplett, 1989). The use of cotton ovule cultures to examine the interaction of cotton ovules with *A. flavus* is simpler than an *in vivo* system as penetration of the carpel wall to inoculate seeds with the fungus may also elicit a plant wound response.

In addition to the heightened production of peroxidase by cotton ovule cultures when challenged by *A. flavus,* other stress-related metabolites are also produced *in vitro*. Within 24 h after inoculation the color of the culture medium is transformed from colorless to brown. Ovules extracted in 85% (v/v) acetone yielded 25 stress metabolites, some of which had antifungal activity. Two of the low molecular weight compounds were identified as the sesquiterpenoid phytoalexins lacinilene C and lacinilene C 7-methylether (Mellon, 1988).

Naturally Pigmented Fiber

In the New World, naturally pigmented cotton fiber has been known since the earliest indigenous people of Central and South America domesticated cotton nearly 5000 years ago. The natural coloration for these fibers may be shades of brown, tan, green, red–brown or gray. In the mid-1980s there was a resurgence of interest in naturally pigmented fibers among fashion-conscious and ecologically minded consumers. As little research had been conducted on genetically improving the naturally pigmented cotton varieties, fiber physical properties such as length and strength were not comparable to those of the white-linted varieties that had been continually improved by plant breeders. This renewed interest in naturally pigmented fibers stimulated research into the biochemistry and physiology of fiber pigmentation.

The naturally green-linted cottons were found to have layers of suberin deposited between the layers of cellulose in the secondary cell wall (Ryser et al., 1983; Yatsu et al., 1983). Cotton ovule cultures have proven useful for monitoring the biosynthesis of compounds associated with this suberin layer. When 2-aminoindan-2-phosphonic acid (AIP), an inhibitor of phenylalanine ammonia-lyase, was added to cotton ovule cultures during the beginning of secondary wall formation, the fibers remained white (Schmutz et al., 1993). In the presence of AIP, two components of green-fiber wax, trans-caffeic acid and an unknown yellow compound, were not synthesized. When S-ethyl-N,N-dipropylthiocarbamate, a specific inhibitor of endoplasmic reticulum-associated fatty acid elongases, was added to cotton ovule cultures the thickness of suberin lamellae was changed (Schmutz et al., 1996). While the structural identity of the compound(s) responsible for the green color of the green-linted varieties has not yet been completely determined, cotton ovule cultures have been an important experimental system for deciphering some of the biochemical pathways involved in the synthesis of these compounds.

The naturally tan, brown and red–brown fiber varieties do not contain the same suberin lamellae in the secondary cell wall that have been characterized in the green-linted varieties. Instead, the unknown compounds providing the color appear to be sequestered in the fiber's large central vacuole. It is surprising that these other naturally pigmented varieties have not been cultured *in vitro* to investigate the biochemistry of their chromogenic compounds.

Cotton Ovule Culture–*G. arboreum* and *G. barbadense*

The original description of culture conditions for cotton ovules by Beasley and Ting and the majority of the remaining studies discussed thus far in this review were conducted with the

predominant cotton variety grown in the United States, *Gossypium hirsutum*. A preliminary report comparing the growth responses of unfertilized and fertilized ovules from *G. hirsutum*, *G. barbadense* and *G. arboreum* on BT medium with and without phytohormones was also made (Beasley and Egli, 1976). The ability to form fiber after 2 wk was evaluated for each of the three species on basal medium without hormones, on BT medium containing 0.5 μM GA_3, on BT medium containing 5.0 μM IAA and on BT medium containing 0.5 μM GA_3 and 5.0 μM IAA. The presence of both phytohormones stimulated the amount of fiber produced by all three species when fertilized ovules were used in culture. The degree of fiber initiation and callus formation from the ovule were variable for the three species, especially when unfertilized ovules were placed on media lacking hormones. Fiber development *in vitro* on *G. barbadense* ovules was highly variable under the phytohormone regimes tested. The authors attributed this variability in response to environmental factors imposed upon the parent plant that 'dictate whether exogenous IAA and/or GA_3 can promote fiber development vs. callus formation' (Beasley and Egli, 1976).

Fiber growth of a temperature-sensitive mutant of *G. arboreum* was also reported (Beasley and Egli, 1977), in which unfertilized ovules on BT medium with phytohormones would produce fiber at temperatures below 30°C and would not produce fiber at temperatures above 32°C. Attempts to repeat these experiments using SMA-2 and SMA-4 germplasm deposited in the USDA-ARS National Cotton Germplasm Collection were not conclusive (Triplett, unpublished results). Slightly over 60% of the SMA-4 ovules produced fiber at the permissive temperature, but 40% of the ovules produced fiber at the restrictive temperature of 32°C.

More recently, the response of *G. barbadense* (Giza 45) ovules in culture was evaluated using a wide range of phytohormone concentrations (Momtaz, 1998). In this study the optimal fiber production *in vitro* by *G. barbadense* resulted when the phytohormones were used at 10 times the phytohormone concentrations used for *G. hirsutum* by the Beasley and Ting protocol. While the endogenous levels of ovular and/or fiber phytohormones have been measured by several investigators (Chen et al., 1996, 1997; Dugger and Sachs, 1987; Gokani et al., 1998; Nayyar et al., 1989), there has never been a direct comparison of phytohormone levels between *G. hirsutum* and *G. barbadense*.

Gravity Effects on Cotton Fiber

A number of studies from both US and Russian space programs concluded that higher plants grown in microgravity have altered plant cell wall structure and composition. As cotton fiber cell walls are not required for the physical support of any plant organ, analysis of the effects of microgravity on cotton fiber development seems like an ideally suited system to define how cell wall biogenesis is affected in the space environment.

Before sending ovule cultures into space, some modifications to the culture conditions were necessary. Normally, in culture, ovules float on the surface of liquid media. Any ovules that sink to the bottom of the culture dish do not develop fiber nor do the ovules grow. Many of the early investigators found that incorporation of agar into culture media either inhibited fiber differentiation or led to prolific callus growth. Recently, our research team has investigated the use of Phytagel™ to solidify ovule culture media (Triplett and Johnson, 1999). The gelling agent had no effect on the length of fibers produced in culture; however, it took 4 wk for fibers grown on solid media to produce the amount of cellulose made in 3 wk by floating ovules. The biochemical basis for the delay in cellulose accumulation in fibers produced on ovules grown on solid media is unknown at this time. Nevertheless, such cultures should prove useful in modeling events leading to differences in fiber wall thickness (maturity) and yield.

The Future

The *in vitro* culture technique first established by Beasley and Ting nearly 30 yr ago continues to be an important research tool for dissecting the complex cellular processes that occur during fiber development. In the near future we will learn the results of several investigators who are using cotton ovule cultures as targets for biolistic transformation to examine transient expression in developing fiber cells. As always, *in vitro* culture of cotton ovules will continue to permit some types of physiological and biochemical experiments that would be difficult with field-grown or greenhouse-grown material, most notably experiments requiring radioisotopes and toxic inhibitors. Nevertheless, it is incumbent on the investigators using cotton ovule culture as a model for fiber development to verify findings with *in planta* grown material to the extent possible. By continuing to establish links between the performance of cotton ovules in culture with ovules developing on whole plants, this important research tool will continue to be used well into the next century.

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